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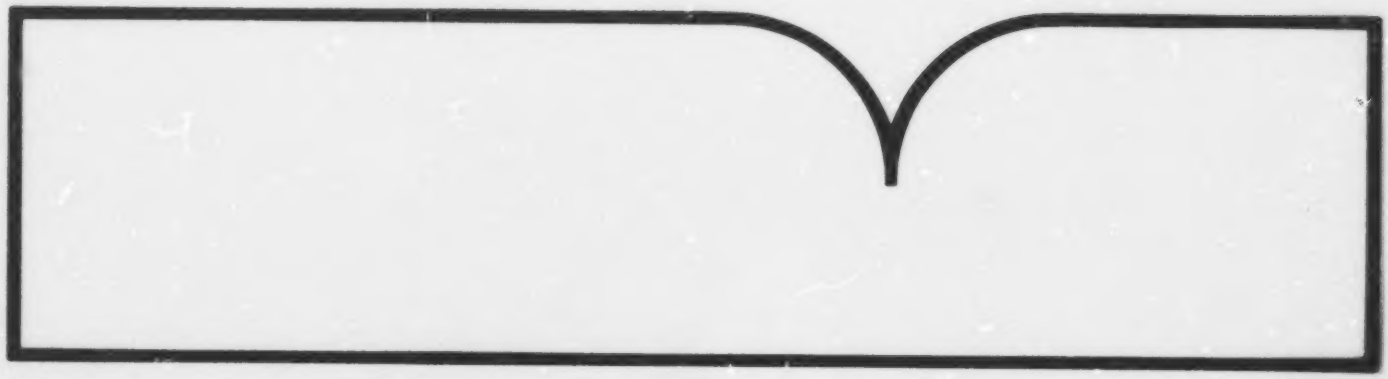

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16. ABSTRACT

Coliforms were enumerated by using m-Endo agar LES and m-T7 agar in 102 routine samples of drinking water from three New England community water systems to investigate the occurrence and significance of injured coliforms. Samples included water collected immediately after conventional treatment, during the backwash cycle, at various points in the distribution system, and 1 week after the break and subsequent repair of a distribution main. Injured coliforms in these samples averaged >95%. m-T7 agar yielded 8- to 38-fold more coliforms than did m-Endo agar LES. The geometric mean of coliforms recovered by m-Endo agar LES was <1 confirmed coliform per 100 ml, although m-T7 agar yielded 5.7 to 67.5 confirmed coliforms per 100 ml. In addition, the majority of these samples giving positive results on m-T7 agar produced no detectable counts on m-Endo agar LES. These findings indicated that coliforms were injured and largely undetected by use of accepted analytical media in the systems examined.

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Injured Coliforms in Drinking Water

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Coliforms were enumerated by using m-Endo agar LES and m-T7 agar in 102 routine samples of drinking water from three New England community water systems to investigate the occurrence and significance of injured coliforms. Samples included water collected immediately after conventional treatment, during the backwash cycle, at various points in the distribution system, and 1 week after the break and subsequent repair of a distribution main. Injured coliforms in these samples averaged >95%. m-T7 agar yielded 8- to 38-fold more coliforms than did m-Endo agar LES. The geometric mean of coliforms recovered by m-Endo agar LES was <1 confirmed coliform per 100 ml, although m-T7 agar yielded 5.7 to 67.5 confirmed coliforms per 100 ml. In addition, the majority of these samples giving positive results on m-T7 agar produced no detectable counts on m-Endo agar LES. These findings indicated that coliforms were injured and largely undetected by use of accepted analytical media in the systems examined.

The coliform group of bacteria has remained the cornerstone of the national drinking water regulations (25) and is used by many in the water supply industry as a criterion of operational parameters. However, some dissatisfaction has been expressed with the shortcomings of reliance upon coliform bacteria as indicators of water quality (7). Some of these concerns have been related to coliform occurrences in the absence of documented waterborne morbidity in the community (4), and others have cited outbreaks of waterborne disease where coliforms were not found (3, 24). The first situation represents a complex, unresolved problem of increasing dimensions that is frequently described as regrowth within the distribution system (4, 10, 18, 22). The latter situation relates to currently accepted methods that lead to underestimations in the detection of waterborne coliforms for a variety of reasons (9, 11, 12, 19, 23). However, the coliform is still regarded as a useful but imperfect criterion of drinking water quality (21, 25; E. E. Geldreich, ASM News 47:23–27, 1981).

A number of chemical and physical factors common to drinking water systems are known to cause a form of sublethal and reversible injury that is responsible for the failure of waterborne coliforms to grow on accepted media used in the analysis of drinking water, such as m-Endo media (11, 14, 19). Factors found in drinking water that can cause injury include chlorine and other biocides, low concentrations of metals such as copper and zinc, extremes of temperature and pH, and interactions with other bacteria (14, 15). After exposure to these stressful factors, injured coliforms are uniquely susceptible to ingredients such as desoxycholate and bile salts that are found in most selective media used to isolate coliforms from water (19). This prompted the development of a selective medium that did not contain bile salts or desoxycholate for the enumeration of injured total coliform bacteria from drinking water (11). The medium was called m-T7. By using this medium and other approaches, surveys were conducted to determine the extent of injury in coliforms found in drinking water from different geographical locations. The results of an early comparative study of samples from community drinking water systems in Montana and Massachusetts by using m-Endo agar LES (Difco Laboratories, Detroit, Mich.) and m-T7 agar with a resuscitation step indicated that approximately half of the coliforms found were injured (14). A later study in Montana, comparing coliform recoveries from drinking water on m-Endo agar LES and m-T7 agar, revealed that 65% were injured (11). These results suggested that the majority of coliforms found in drinking water were injured. However, questions about the universality of that hypothesis remained, because injury results from the collective influence of many factors (6, 20, 21) that may be present in various levels in drinking water from different regions.

This study was initiated to learn more about the occurrence of injured coliforms and their significance in community water systems. Routine samples of drinking water from three New England water systems experiencing chronic or sporadic occurrences of coliform bacteria were analyzed for coliforms with m-Endo agar LES and m-T7 agar. The samples included water collected both during and immediately after conventional treatment, during the backwash cycle, and at various points in the distribution system, including 1 week after the break and subsequent repair of a distribution main. The results revealed that >90% of the coliforms isolated were injured. Recovery of confirmed coliforms on m-T7 agar was 8- to 38 times higher than that on m-Endo agar LES. In addition, the majority of samples analyzed on m-Endo agar LES yielded negative results, although confirmed coliforms were isolated by using m-T7 agar. These findings indicate that coliforms in routine distribution water samples, chlorinated water leaving treatment plants, and water associated with broken and repaired pipes are frequently undetected by accepted enumeration procedures. Further, these results have important consequences for drinking water systems experiencing coliform regrowth problems.

MATERIALS AND METHODS

Study sites and sample collection. Water samples were collected from various points within the drinking water treatment facilities and distribution systems of three New England communities. The systems studied were located at Salem and Beverly, Mass.; Bennington, Vt.; and Kennebunk,

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TABLE 1. Detection of injured coliforms in three New England drinking water treatment and distribution systems

Sample no.	Sample source	No. of samples	No. of confirmed colonies/100 ml detected on:		% injury	% false-negative
			m-Endo agar LES	m-T7 agar		
1	Throughout systems	71	0.3	9.5	96.8	79
2	Water leaving treatment plants	46	0.2	5.7	96.5	69
3	Filter backwash	1	18	136	86.7	
4	After backwash	1	5	42	97.4	82
5	Pipe break	28	0.9	35.3	97.4	82
6	1 wk after pipe break	11	0	67.5	100	100
7	After disinfection of new main	1	0	11	100	100

Maine. All three of these systems have experienced chronic or intermittent occurrences of excessive coliform populations in the past, including the time this study was conducted.

The system at Salem and Beverly used surface water from a lake and reservoirs that was conventionally treated by using aluminum sulfate, lime, and a phosphate-based corrosion inhibitor. The water was chlorinated before and after rapid sand filtration to maintain a free-chlorine residual concentration of approximately 1.0 mg/liter, although no chlorine was detected in some dead-end samples. This system served a population of approximately 75,000. Bennington received water from a brook in an agricultural watershed. Conventional treatment without prechlorination was followed by chlorination to a free-chlorine residual concentration of 0.5 mg/liter. This system served a population of approximately 16,000. The Kennebunk water district also used conventional treatment, with alum coagulation, soda ash, and a phosphate-based corrosion inhibitor. Pretreatment and postreatment chlorination to a level of 1.0 mg of free-chlorine residual per liter was practiced, but that concentration was not always found in some outlying areas of the distribution system.

Water samples were collected in 250-ml glass or polypropylene bottles with added sodium thiosulfate (0.008%) plus EDTA (1). Free and total chlorine levels were measured at the time of sampling by using a chlorine kit (DPD; Hach Chemical Co., Loveland, Colo.). Samples were placed on ice or in a cooler and transported to the laboratory, where most were analyzed within 4 h after collection. Samples from Bennington were analyzed within 12 h because of shipping requirements.

Microbiological analyses. Comparative analyses for total coliform bacteria were performed on each water sample by using m-Endo agar LES and m-T7 agar. m-Endo agar LES was prepared according to the specifications of the manufacturer. m-T7 agar was prepared as described, including penicillin, by LeChevallier et al. (11). Sample volumes of 100 ml each were filtered through membrane filters (HA WG 04721; Millipore Corp., Bedford, Mass.) and incubated at 35 ± 0.5°C. Sheen colonies on m-Endo agar LES and yellow colonies on m-T7 agar were counted by using a magnification of ×15 according to established guidelines (1, 11). Positive colonies were confirmed by Gram stain and the β-galactosidase-cytochrome oxidase method (1, 12). Additionally, approximately one-third of the confirmed colonies from both media were identified with the API 20E system (Analytab Products, Plainview, N.Y.).

Quality control and statistical comparisons. Accepted quality assurance practices (1, 2) were observed throughout this study. Statistical comparisons were made by using the paired *t* test on logarithmically transformed data.

RESULTS

Water samples collected from various locations within three drinking water treatment and distribution facilities in New England were analyzed for total coliform bacteria by using m-Endo agar LES and m-T7 agar. m-Endo agar LES was used because m-Endo media are most frequently applied in the enumeration of coliforms in drinking water in the United States (1). m-T7 agar was selected because it allows the resuscitation and recovery of damaged cells (11). Therefore, a comparison of the resulting data provided an opportunity to examine the occurrence of injured coliforms in operating drinking water systems, the utility of m-T7 agar, and the significance of injured coliforms in drinking water systems having chronic occurrences of indicator bacteria in three drinking water systems. The results show the comparative recovery of coliforms in 102 water samples from the three systems studied (Table 1). Results from a subset of 71 routine samples obtained from throughout the distribution systems revealed that a major portion (96.8%) of the confirmed coliforms recovered from finished drinking water were injured and not enumerated as either typical or atypical colonies on m-Endo agar LES (Table 1). The remainder of the sample categories, likewise, showed injury ranging from 86.7 to 97.4% (Table 1). It should also be noted that m-Endo agar LES detected no coliforms in 78% of samples showing positive results on m-T7 agar. Also, the mean coliform level determined with m-Endo agar LES was less than 1.0 confirmed coliform per 100 ml for most of the samples, although it ranged from 5.7 to 67.5 confirmed coliforms per 100 ml for m-T7 agar. The differences observed in the coliform enumerations with m-Endo agar LES and m-T7 agar were highly significant for all data sets ($P < 0.001$). Only 9 of the 102 samples analyzed yielded no detectable coliforms on both m-Endo agar LES and m-T7 agar.

Finished drinking water leaving the treatment plants was also examined to determine if injured coliforms were passing undetected into the distribution system. Results of the 46 sample subsets of treated chlorinated water immediately after filtration are shown in Table 1. As before, a high percentage (96.5%) of the coliforms were injured. The mean coliform level determined with m-Endo agar LES was less than 1.0 confirmed coliform per 100 ml, although it was much higher (5.7 confirmed coliforms per 100 ml) when enumerated with m-T7 agar. Additionally, 69.5% of the samples had positive results on m-T7 agar but failed to give any indication of coliforms on m-Endo agar LES. The total chlorine con-

centration of the water in the filter was maintained near 1.4 mg/liter. Similar results were seen in the two samples taken during and immediately after one backwash cycle of a sand filter (Table 1).

In January 1985, a distribution pipe (12 in. [30 cm] in diameter) in the Salem and Beverly system ruptured and was repaired. This break resulted in reduced chlorine levels and the occurrence of elevated numbers of coliforms in the drinking water. A summary of the resulting bacteriological data from the 2 weeks after this event are shown in Table 1. The mean coliform counts again showed a large difference between the two media and a high degree of coliform injury (97.4%). In four of these samples, the confirmed coliform count on m-T7 agar was in excess of 1,500 confirmed coliforms per 100 ml. The isolated bacteria were identified as *Klebsiella oxytoca* and *Enterobacter agglomerans*. During this time, the total (0 to 0.5 mg of chlorine per liter) and free (0 to 0.4 mg of chlorine per liter)-chlorine levels were lower than those normally observed within the system. Of particular interest are samples that were obtained in the same location 1 week after the rupture (Table 1). All 11 samples failed to produce coliform colonies on m-Endo agar LES but yielded a mean coliform count of 67.5 confirmed coliforms per 100 ml on m-T7 agar. A single sample obtained after the replacement of a distribution pipe (8 in. [20 cm] in diameter) disinfected for 24 h with 200 mg of chlorine per liter and flushed before being placed in service, showed similar results (Table 1). Here again, m-Endo agar LES failed to yield any coliform colonies, although m-T7 agar revealed 11 confirmed coliforms per 100 ml. These organisms were confirmed and identified as *E. agglomerans*.

Confirmed coliforms were identified in 27 of the samples analyzed. The majority (84%) of these isolates were *Enterobacter aerogenes*, *E. agglomerans*, and *Klebsiella pneumoniae* (Table 2). In addition, the bacteria isolated on the two media were identical with respect to organisms found and their relative abundance.

DISCUSSION

The coliform indicator concept has been useful in helping to provide safe drinking water despite its imperfections (21). Properly designed analytical programs, carefully executed to detect coliform bacteria within drinking water systems, have been of value in monitoring the effectiveness of treatment practices as well as the intrusion of contaminated water. However, within the past 20 years, the incidence of waterborne morbidity has increased steadily in the United States (17). The causes of this trend are complex and not completely understood, but excessive populations of coliforms have been associated with most of these outbreaks that have been investigated (5), suggesting that this group of bacteria can still provide useful information in many of the instances of waterborne disease (21). One important application of such information is to guide remedial action when defects of malfunctions arise in treatment and distribution systems. The majority of waterborne disease outbreaks have been caused by problems within the system or an interruption of some aspect of the treatment process (17). Therefore, it is important to optimize coliform detection by making the analysis more sensitive to maintain a commitment to high-quality drinking water.

TABLE 2. Identification of coliforms isolated from three New England drinking water systems by using m-Endo agar LES and m-T7 agar[a]

Organism	No. of isolates	% of total isolates
E. agglomerans	26	33
K. pneumoniae	21	27
E. aerogenes	19	24
E. cloacae	5	7
K. oxytoca	4	5
Citrobacter freundii	3	4

[a] Bacteria were identified from 33 of the 102 samples tested.

Recent reviews (13, 14) describe causes, implications, and methods for the enumeration of injured coliforms in drinking water, thus, those topics will not be discussed in detail here. However, the failure to detect injured coliforms in water implicated in waterborne disease outbreaks (3, 24) is an example of how injured coliforms may be of public health importance. This possibility is further supported by the recent finding by LeChevallier et al. (16) that waterborne pathogenic bacteria are more resistant to injury by chlorine than are similarly exposed coliforms. These results support the view expressed by Seidler (23) that methodological inadequacies in the enumeration of coliforms are basic to some of the dissatisfaction (7) with the indicator concept applied to the microbiological analysis of drinking water.

Available data concerning the occurrence and significance of injured coliforms in drinking water are limited. This paucity of data is caused by the relatively new concept of injury to coliform bacteria in drinking water as well as the lack of suitable commercial media to recover injured bacteria from environmental samples. However, the development of m-T7 agar (11) provided an advance in this regard, because it is both selective and differential and was formulated specifically for the enumeration of injured coliforms from drinking water. This advance paralleled efforts by others to improve the sensitivity of most-probable-number and confirmation methodologies (12, 23). A survey of 44 chlorinated drinking water samples from communities in Montana showed that m-T7 agar recovered nearly three times more coliforms than did m-Endo agar LES (11). Additionally, an earlier survey of over 200 chlorinated and unchlorinated drinking water samples from Montana and Massachusetts, by using the standard m-Endo agar LES alone and with a resuscitation step, revealed that in 31 samples containing coliforms, coliform injury levels ranged from 31 to 86% with a mean level of 43% (unpublished data). In another unpublished study, investigators at a system in Southern California examined 28 replicates of four drinking water samples with both m-Endo agar LES and m-T7 agar and found 58.5% injury in the coliforms that were detected. The present study was initiated to extend this body of knowledge by investigating the occurrence and significance of injured total coliform bacteria found in drinking water systems experiencing excessive coliform populations as well as to evaluate m-T7 agar in other geographical locations. The results show that a high percentage (86.7 to 97.4%) of the coliforms present in the three systems studied were injured (Table 1). This indicates that approximately 1/10 of the coliforms present were enumerated when the accepted method with m-Endo agar LES was used. Whether this level of cellular damage is found in all systems is uncertain, because injury results from the collective influence of chemical and physical properties of water (20) that vary markedly in different regions and systems (21). In fact, drinking water distribution samples that were similarly evaluated from a municipal system in New Jersey experiencing an occurrence of coliforms revealed little difference between enumerations with m-Endo agar LES and m-T7 agar with a mean coliform

injury level of only 13% (unpublished data). However, in all cases where we have seen this kind of comparative data from drinking water, m-T7 agar has been as effective as or superior to other media in enumerating total coliform bacteria.

Occurrences of excessive coliform populations in drinking water, termed regrowth, are being observed with increasing frequency (4, 10, 18, 22). The source of these coliforms in the distribution water is presumed, in the absence of definitive data, to be growth within a biofilm community on the pipe walls (4, 18, 22). Geldreich et al. (9) have suggested that coliforms from sand filters "seed" the distribution system and demonstrated that filters contain between 110 (deep) and 6,300 (surface) coliforms per gram of filter-bed sand. More recently (10), investigators at Springfield, Ill., proposed that chlorine-injured coliforms passed undetected into the distribution system, where they were able to recover and cause a problematic coliform occurrence. The results reported here for both filter effluents and samples taken during and immediately after a backwash cycle lend support to the first part of that scenario (Table 1). Further, it is useful to note that problem coliform occurrences in systems with surface source water usually follow major precipitation events when the water temperature is relatively warm (18, 22). The precipitation might serve to wash coliforms from the environment, where they are known to proliferate (8), into the source water along with added nutrients that allow them to grow on the filter. Chlorine and other injurious factors may damage such bacteria, making them undetectable with m-Endo media as they are released into the distribution system, where the temperature and added nutrients favor their recovery.

The results reported here address unexplained occurrences of coliforms within distribution systems (Table 1). Our findings demonstrate that a high percentage (96.5%) of the coliforms present in the chlorinated water immediately after filtration are injured and not detected with m-Endo agar LES. For that reason and because m-Endo agar LES is the accepted membrane filter medium, injured coliforms entering the distribution network are frequently not observed. That argument is further supported by the high percentage (78%) of samples with coliforms on the m-T7 agar plates that failed to yield colonies on m-Endo agar LES.

Another feature of the overall data presented in Table 1 is noteworthy. In 100 of the 102 samples examined, excluding those associated with the backwash of a sand filter, the geometric mean values of coliform enumerations with m-Endo agar LES were in compliance (0.2 to 0.9 confirmed coliform per 100 ml) with the national drinking water regulations (<1 confirmed coliform per 100 ml) (25), although they far exceeded that standard when m-T7 agar was used (5.7 to 67.5 confirmed coliforms per 100 ml) if those values were monthly averages. In such systems, therefore, the use of a more efficient medium such as m-T7 agar would afford greater sensitivity in the microbiological aspect of the routine water analysis and allow emerging problems to be detected earlier, because many such operational or intrusion difficulties are first signaled by a low level of coliforms that increases numerically until the system is no longer in compliance with the coliform limit specified in the regulations. Clearly, the operator of such a system would want to know of the impending problem as early as possible to initiate the appropriate corrective action. Hence, use of the more sensitive m-T7 agar would be preferred, because it would provide a more accurate understanding of the source of coliforms entering the distribution system as well as their location and population dynamics. This same interpretation might be extended to the finding of high percentages of samples that revealed no coliforms with m-Endo agar LES but showed positive results on m-T7 agar; these ranged from 69.5 to 100% of the samples within each data set (Table 1). Results describing the observation of injured coliforms associated with the rupture and repair of a distribution main also support the same line of reasoning (Table 1). Data from the entire 2-week period after this event again show a high degree of injury with the geometric mean for the coliforms detected with m-Endo agar LES at a level (0.9 confirmed coliform per 100 ml) that is less than the national coliform standard for drinking water (Table 1). Even more striking are the data from the samples collected 1 week after the break and repair event (Table 1). High coliform counts were seen with m-T7 agar (mean of 67.5 confirmed coliforms per 100 ml), although the samples were universally negative when enumerated with m-Endo agar LES.

The following conclusions are proposed concerning the occurrence, detection, and significance of injured coliform bacteria in the three New England municipal drinking water systems studied. (i) The coliform bacteria present in these systems were injured to the degree that accepted methods, by using m-Endo agar LES, would enumerate less than 1/10 of the viable population present. This caused 70 to 100% of the samples in the data sets examined to yield false-negative results, a finding of significance when considering presence-absence methodologies. (ii) m-T7 agar was effective in the recovery of the injured portion of the total coliform population yielding results that were 8- to 38 times greater than those with m-Endo agar LES. (iii) Significant levels of injured coliforms were undetected entering the distribution system after treatment, including filtration, and after the repair and disinfection of a broken main if m-Endo agar LES was used. These findings may not be universal but may be characteristic to some geographic regions or to particular drinking water systems where the physicochemical properties of the water induce coliform injury. In systems such as the ones described here, injured coliforms can represent the majority of the total coliform population present. Optimal enumeration of these bacteria with more sensitive media, such as m-T7 agar, provides those individuals concerned with the maintenance of high-quality drinking water a more useful and representative body of water quality information.

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